Acta Haematologica

Acta Haematol 1999;101:89–96

Gene Therapy for Severe Combined Immunodeficiency Caused by Adenosine Deaminase Deficiency: Improved Retroviral Vectors for Clinical Trials

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Key Words
Gene therapy · Retroviral vector · Severe combined immunodeficiency, adenosine deaminase · Stem cells, hematopoietic · T lymphocytes, peripheral

Abstract
Severe combined immunodeficiency (SCID) caused by adenosine deaminase deficiency (ADA–) is the first genetic disorder to be treated with gene therapy. Since 1990 when the first trial started for 2 patients with ADA–SCID, five clinical trials enrolling 11 patients have been conducted with different clinical approaches and the results obtained from these trials have recently been reported. According to these reports, T cell-directed gene transfer was useful in the treatment of ADA– SCID whereas the retroviral-mediated gene transfer to hematopoietic stem cells was insufficient for achievement of clinical benefits. This chapter reviews several crucial problems inherent in the current retroviral technology based on the clinical data observed in these pioneering ADA gene therapy trials and presents our new retroviral vector system for the next stem cell gene therapy.

Introduction

Advances in molecular biology for the last 3 decades have demonstrated the feasibility of a human gene therapy approach in which the introduction of a therapeutic gene could correct patient cell function and mitigate disease [1]. Since 1983 when the first successful gene transfer with a retroviral vector to murine hematopoietic cells was reported [2], a number of experiments using murine hematopoietic stem cells have been conducted to assess the possibility of human stem cell gene therapy. In recent years, murine in vivo studies have shown that recombinant murine retroviruses are able to infect murine hematopoietic stem cells with high efficiency and sustain a long-term expression of the transduced gene [3]. By extrapolating the success of these murine studies, it was believed that human stem cell gene therapy would soon be applicable to treat a wide variety of congenital or acquired human diseases such as cancer and acquired immune deficiency syndrome (AIDS). Since the first clinical gene therapy trial started in 1990 for 2 patients with adenosine deaminase deficiency (ADA–) [4], many clinical trials using human hematopoietic stem cells have been per-

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formed. However, results obtained from these trials revealed that such an extrapolation was not justified [5, 6]. The transduction efficiency in human hematopoietic stem cells was disappointingly low and sustained long-term expression of the transgene was not observed. Furthermore, some patients mobilized immune responses against the dominant selectable markers which could eliminate the cells expressing a therapeutic gene [7, 8].

Here we will highlight the problems inherent in the current retroviral technology by discussing gene therapy clinical trials for ADA– severe combined immunodeficiency (SCID). We will also present our new strategy including new retroviral vectors for the next clinical trial.

ADA Deficiency

ADA (EC 3.5.4.4) is a critical enzyme in the purine salvage pathway catalyzing the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively [9]. The catalytic activity of the enzyme resides in a single polypeptide encoded by a locus on the long arm of human chromosome 20. Molecular weights of 38,000–44,000 daltons have been reported for the polypeptide. The absence of ADA activity leads to an accumulation of deoxyadenosine which is converted to the phosphorylated form (deoxyadenosine triphosphate, dATP) in vivo. Since lymphocytes, and thymocytes in particular, are very sensitive to the toxic effects of dATP, inherited deficiency of ADA impairs the function of the immune system resulting in SCID characterized by severe T lymphocyte dysfunction and agammaglobulinemia. The current curative treatment of choice for ADA– SCID is bone marrow transplantation (BMT) from HLA-identical siblings, which restores more than 90% of the patients to complete immune function [10]. However, less than one third of the patients have access to an appropriate donor and BMT from haploidentical donors (the patient's parents) is generally less successful. An alternative is enzyme replacement using polyethylene glycol-modified bovine ADA (PEG-ADA), which leads to improvement of lymphocyte count and function [11]. PEG-ADA is considered to be a life-saving, but costly, therapeutic option for patients that do not have an HLA-matched donor. However, a few patients have been unresponsive to PEG-ADA and development of an antibody against the bovine peptide has often been described.

Gene Therapy Clinical Trials

A number of rational reasons have made ADA– SCID the first target disease of gene therapy. First, ADA deficiency is the most extensively studied of all the congenital immunodeficiency diseases because the genomic and cDNA sequences encoding ADA were identified early [12–15] and the function of the enzyme is well understood. Second, tight regulation of ADA expression is not necessary to restore the patient's immune functions and suppress adverse effects because patients with 10% of normal ADA levels do not show any apparent immune impairments [16]. Conversely, kindred with ADA activity greater than 50-fold above normal have only a moderate hemolytic anemia [17]. Third, genetically corrected cells should have a selectable growth advantage over the nontransduced cells in vivo. Fourth, expression of exogenous ADA will restore the patient's immune function as demonstrated in the PEG-ADA replacement therapy.

In 1990 Blaese's group [18] at the National Institutes of Health (NIH) performed the first clinical gene therapy trial for 2 patients with ADA– SCID. In the pioneering trial they adapted the patients' peripheral T lymphocytes as a target cell population based on two important findings. First, preclinical attempts to use hematopoietic stem cells in a nonhuman primate model system prior to the trial resulted in only low-level, transient gene expression and proved to be insufficient for clinical use [18]. Second, the only surviving donor cells present in some patients cured by allo-BMT were observed to be T cells [19]. In the trial, patient peripheral lymphocytes obtained by apheresis were stimulated with the interleukin-2 (IL-2) and anti-CD3 antibody (OKT3) for 72 h, transduced with the retroviral vector LASN containing the ADA gene, expanded >50-fold, and infused back into the patients. The patients received a total of 11–12 infusions of autologous genetically corrected lymphocytes over 2 years. As a result, immune functions in both patients improved to levels which had been not observed during the period of PEG-ADA-only treatment and have remained stable more than 4 years since the discontinuation of gene therapy.

The success of this pioneering research encouraged us to start the first clinical Japanese gene therapy trial for an ADA– SCID boy following the identical protocol used in the NIH trial [20]. The Japanese study allowed an additional opportunity to evaluate the efficacy and safety of peripheral T lymphocyte-directed gene therapy for ADA–SCID. The patient was a 5-year-old boy diagnosed with 'delayed-onset' ADA– SCID at 12 months of age. Since no

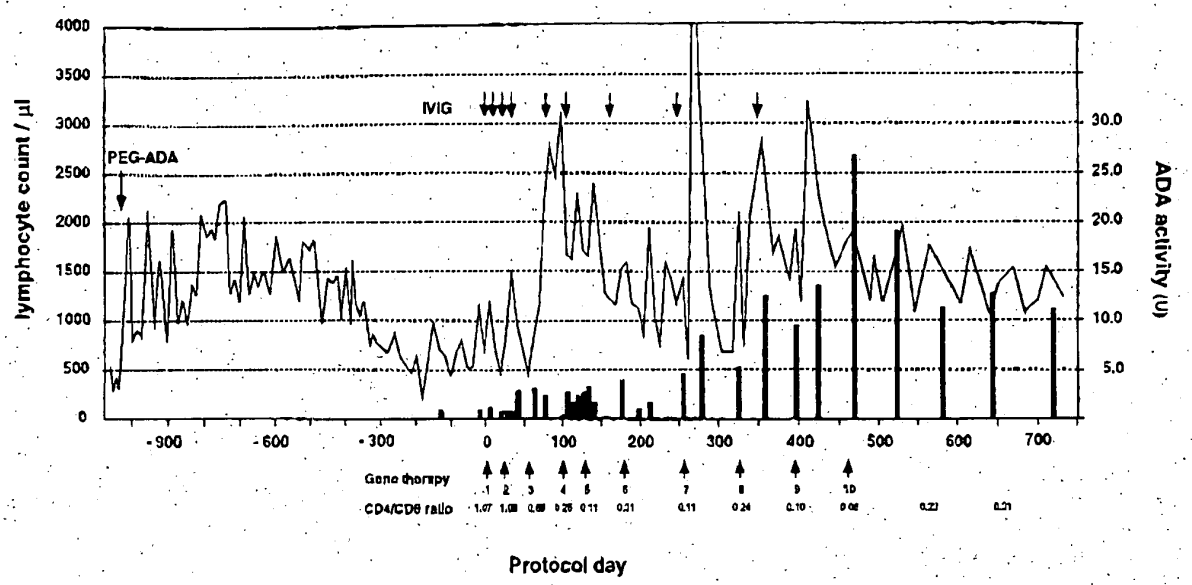

Fig. 1. Clinical course of the Japanese ADA– SCID patient before and after gene therapy. The patient received a total of 10 infusions of genetically corrected autologous T lymphocytes over 18 months. The first infusion was in August 1995 (protocol day 0) and the last (the 10th infusion) was in March 1997 (protocol day 462). PEG-ADA therapy was initiated at 15 months of age. The lymphocyte count is indicated by the solid line and the CD4/CD8 ratio was measured using the patient's peripheral lymphocytes before infusion. ADA enzyme activity shown by solid bars is expressed as nanomoles of inosine and hypoxanthine produced per minute by 10^8 cells. Arrows show replacement of IVIG after gene therapy. The patient received Ig replacement (2.5 g) monthly before gene therapy.

suitable bone marrow donor was available, PEG-ADA treatment was initiated at 15 months of age. Despite continuous PEG-ADA treatment, however, his serum Ig levels remained below normal and lymphopenia recurred during the second year of enzyme replacement. The patient had received a total of 10 infusions of genetically corrected peripheral lymphocytes over 18 months (fig. 1). After initial fluctuation, the patient's T cell counts stabilized in the normal range and have been sustained over 12 months after cessation of the infusions. Progressive inversion of CD4/CD8 has been observed since the 4th infusion due to an increase of the absolute CD8+ cell count. This phenomenon is thought to be the result of preferential proliferation of CD8+ cells during in vitro culture and transduction. The patient's cell-associated ADA enzyme activity increased from barely detectable before treatment and remained at half of the values found in peripheral mononuclear cells of his heterozygous carrier mother. The interval between intravenous Ig (IVIG) infusions which had been given monthly before gene therapy was widened and eventually stopped at the 8th infusion. Despite this, the patient's serum Ig levels gradually increased and have remained normal over 1 year without additional IVIG treatment. The frequency of integrated provirus in the patient's peripheral blood has remained stable at 15% (0.1–0.2% proviral copies/cell) since the 4th infusion. Eleven months after beginning gene therapy, the patient's isohemagglutinin titers (IgG) increased from undetectable to 1:16 and delayed hypersensitivity skin tests, a measure of T cell function, became stronger. The patient has gained 3 kg in weight during this trial. He is still receiving periodic PEG-ADA replacement and is attending a public school with isolation precautions.

Together with the observation made in the NIH trial, our trial strongly indicates that T lymphocyte-directed gene therapy is a viable addition to treatment options to

be considered for ADA– SCID patients without suitable bone marrow donors. However, it should be noted that periodic monitoring and further infusions of genetically corrected T lymphocytes are expected to be required in the future to keep the patient's immune function normal due to the finite life span of T lymphocytes. Furthermore, these repeated infusions may induce host immune reactions as reported in other patients, resulting in elimination of the successfully transduced cells in vivo [8].

From this perspective, stem cell gene therapy is the most attractive for ADA– SCID since only one infusion of genetically corrected stem cells may restore the patient's immune functions as demonstrated by allogeneic BMT. Three trials have been undertaken to assess the possibility of treating ADA– SCID patients by correcting hematopoietic stem cells. Brodignon's group [21] transduced the patient's bone marrow cells as well as peripheral lymphocytes with two different ADA vectors and reported rapid improvement of the patient's immune functions after gene therapy. Interestingly, while the peripheral lymphocytes which had been transduced with the ADA gene initially supplied the peripheral functional T cell population, they were later replaced by cells derived from the transduced bone marrow cells. This suggests that the genetically modified hematopoietic stem cells are able to give rise to functional mature cells. On the other hand, the results obtained from two other trials were unsatisfactory. The European group using CD34+ cells from bone marrow as a target cell population reported that the transduction efficiency was disappointingly low and the transduced cells could be detected only within 6 months [22]. This group emphasized the importance of myeloablation in stem cell gene therapy. Kohn's group [23] started gene therapy using CD34+ cells obtained from cord blood as a novel target cell for the treatment of acquired diseases such as cancer, HIV infection, and congenital disorders. Although cord blood proved more susceptible for efficient retroviral-mediated gene transfer compared with bone marrow, it was clear that significant advances were still needed in gene transfer and expression technology to achieve clinical benefits [24].

Improved ADA Retroviral Vectors for Clinical Trials

A number of efforts have been directed toward the identification of factors regulating hematopoiesis and retrovirus infection including improving retroviral vector constructs and packaging cell lines. Knowledge obtained from these trials has been applied in clinical trials. In this study, we made new ADA retroviral vectors that are more effective than LASN/PA317 currently used in clinical trials by removing dominant selectable markers and by using different packaging cell lines [25].

Retroviral Vector Constructs

A number of considerations was incorporated into newly constructed vectors (fig. 2a). First, the vectors were simplified by removing dominant selectable markers since proteins of nonhuman origin sometimes induce strong host immune reaction. Second, the vectors have a splice acceptor sequence to allow production of subgenomic RNA which can be translated with high efficiency. Third, the ADA cDNA was cloned into the vectors such that the ADA translational start site was at the precise location of the env translational start site used in the wild-type virus. Fourth, a series of vectors containing different LTRs were engineered to compare the levels of expression from the LTRs in relevant target cell populations (fig. 2b). The LTRs chosen were from Molony murine leukemia virus (MLV), myeloproliferative sarcoma virus (MPSV) and SL3-3. MPSV or SL3-3 is known to be a strong promoter/enhancer unit in immature cells such as hematopoietic progenitors or T cell lineage, respectively. These vectors were compared with LASN which has ADA expressed from the MLV LTR and neo-driven by the SV40e promoter [26]. All the vectors were packaged in the PG13 packaging cell line with the gibbon ape leukemia virus envelope [27].

Limiting dilution and RNA dot blot analysis [28] allowed identification of the highest titered PG13 clones for LASN, MLV, MPSV and SL3-3. The clinical vector PA317/LASN was used as a control. The relative titers of these clones estimated by the RNA dot blot analysis and G418 selection are as follows (from lowest to highest): PG13/ADA(MLV), PA317/LASN (1×10^4 CFU/ml on HeLa cells), PG13/ADA(SL3-3), PG13/LASN (4×10^5 CFU/ml on HeLa cells), and PG13/ADA(MPSV). Southern blot analysis of all the retroviral producer cell clones revealed full-lenght proviral integrants, suggesting no gross rearrangements or deletions.

Transduction to ADA– Patient's Hematopoietic Cells

To assess the ability of these clones to transduce the ADA gene into the patient's primary peripheral lymphocytes, peripheral mononuclear cells isolated from an ADA– SCID patient by apheresis were transduced with equal volumes of virus supernatants from these clones after prestimulation with OKT3 and rIL-2 for 4 days to

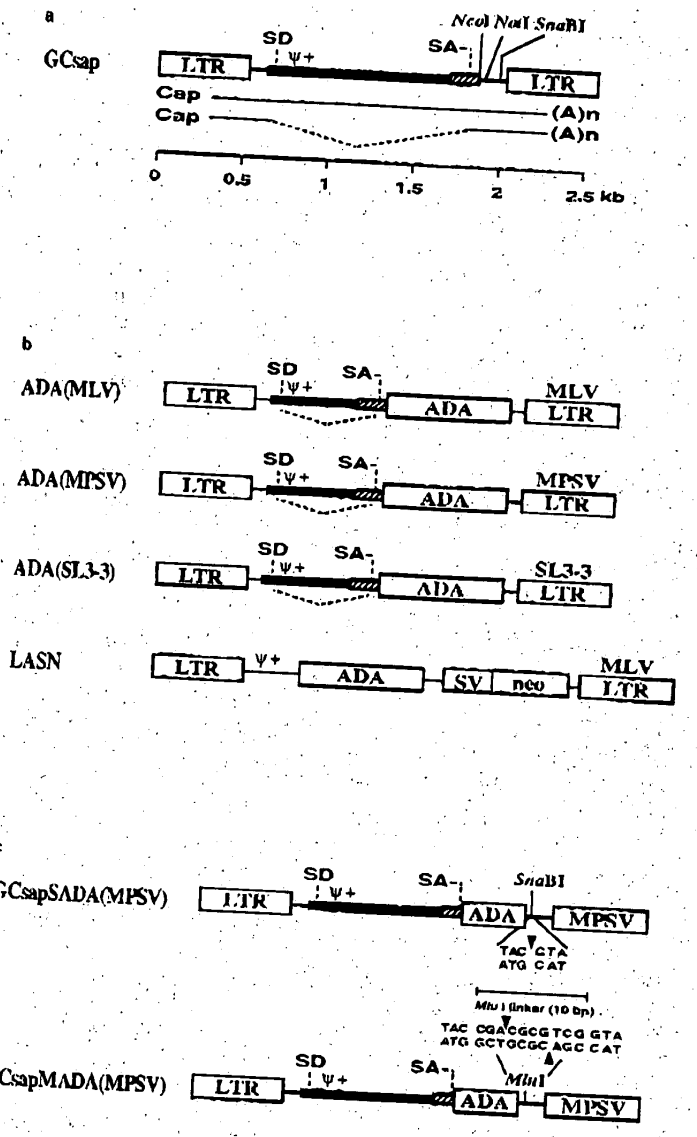

Fig. 2. Structure of the simplified retroviral vector GCsap and the derivative ADA vectors. GCsap has the MLV LTR with intact SD and SA sites (**a**). ADA cDNA (ADA) was cloned between *Nco*I and *Not*I sites of GCsap to generate ADA(MLV). The 3'MLV LTR was replaced by the corresponding MPSV or SL3-3 fragment to make ADA(MPSV) or ADA(SL3-3), respectively (**b**). *Mlu*I linker was incorporated into *Sna*BI site of ADA(MPSV) to make GCsapMADA(MPSV). Therefore, ADA (MPSV) is referred to as GCsapSADA (MPSV) (**c**). Ψ+ = Packaging signal; SV = SV40 early promoter; neo = neomycin phosphotransferase gene.

mimic the clinical trials. After an expansion period of 7 days, the proviral copy number and ADA enzyme activity in the transduced cells were analyzed. A semiquantitative PCR analysis showed that the ratio of vector DNA per genomic DNA observed in PG13/ADA(MPSV) exceeded that observed in 100% LASN, suggesting that transduction frequency of PG13/ADA/(MPSV) is higher than 1 copy/cell in primary peripheral lymphocytes (fig. 3). Consistent with the PCR data, PG13/ADA(MPSV) showed the highest ADA enzyme activity in the transduced lymphocytes and the value was approximately 16-fold higher than that observed in the clinical vector PA317/LASN (table 1).

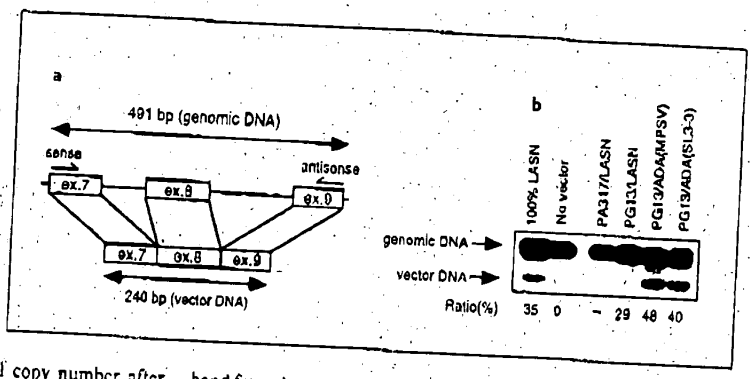

Fig. 3. PCR analysis to evaluate the proviral copy number after retroviral transduction of peripheral mononuclear cells from an ADA– SCID patient. PCR amplification with the sense and antisense primers (**a**) yields two bands in cells containing proviral integrants: a 491-bp band from the endogenous ADA gene and a 240-bp band from the proviral integrant. **b** The ratio values were determined by dividing the phosphoimager signal of the vector DNA band by that of the genomic DNA band. LASN-transduced, G418-selected HeLa cells (100% LASN) were used as the 100% transduced standard. ex = Exon.

Table 1. ADA enzyme activity in the transduced patient's peripheral blood mononuclear cells

	Experiment 1	Experiment 2	Average
Untransduced	2	0	1
PA317/LASN	36	27	32
PG13/LASN	237	186	212
PG13/ADA(MPSV)	461	568	515
PG13/ADA(SL3-3)	80	79	80
Normal PBMC	82	85	84

ADA enzyme activity is expressed as nanomoles of inosine produced per minute per 10^8 cells. PBMC = Peripheral blood mononuclear cells.

Comparison of Different Packaging Cell Lines

Having identified ADA(MPSV) as the best vector, the next step was to determine the best packaging cell lines for gene therapy. Recently, several packaging cell lines of human origin which generate retrovirus resistant to human complement became available. FLYA13 and FLYRD18 derived from human fibrosarcoma HT1080 are engineered to express the murine amphotropic env and feline virus RD114 env, respectively [29]. After identification of the highest titer clones of FLYA13/ADA(MPSV) and FLYRD18/ADA(MPSV) by the dot blot analysis, their ability to infect the ADA– patient T cell line TJF-2 [30] was compared with PG13/ADA(MPSV). The results of transduction experiments indicated that both FLYA13 and FLYRD18 outperformed the PG13 clone for ADA expression in the transduced TJF-2 and FLYRD18/ADA(MPSV) provided a 17-fold-higher level of ADA expression than the clinical vector PA317/LASN (table 2). This was consistent with Southern blot analysis of the transduced cells, which demonstrated that the FLYRD18 vector gave the highest relative proviral copy number. Interestingly, all producer cell lines yielded similar levels of ADA enzyme activity per one copy of virus since expression was driven by the same promoter (MPSV LTR) in all cases (experiment in 3 in table 2).

Virus infection was partly determined by interaction between the viral envelope and the virus receptor on the surface membrane of the target cells. To determine the viral envelope suitable for human hematopoietic stem cells, CD34+ cells obtained from cord blood were transduced with a neomycin phosphotransferase gene (neo) containing vector (G1NS) produced by the four packaging cell lines PA317, PG13, FLYA13 and FLYRD18. At 2 days after transduction, the cells were plated in methylcellulose containing rIL-3, GM-CSF, stem cell factor and Epo with or without 1 mg/ml G418. At the 12th day of culture, the colony number was counted. The transduction frequency was determined by division of the total colony number in G418-containing culture by that in G418-free culture (table 3). The highest transduction frequency was observed in PG13 clones in the first two experiments in which all the supernatants were diluted to

Table 2. ADA enzyme activity in the transduced TJF-2

	TJF-2			
	experiment 1	experiment 2	experiment 3	average
Untransduced	4	4	3	4
PG13/ADA(MPSV)	138	100	98 (1.0)	112
FLYA13/ADA(MPSV)	68	206	232 (2.0)	167
FLYRD18/ADA(MPSV)	323	308	422 (4.8)	351*

ADA enzyme activity is expressed as nanomoles of inosine produced per minute per 10^8 cells. Average proviral copy number/cell is given in parentheses. * $p < 0.05$ compared with PG13/ADA(MPSV).

3×10^5 CFU/ml. In experiment 3, undiluted supernatants of these clones were used for transduction. While the transduction frequency in PA317 clones increased 3 times accompanied with an increase of virus titer, little increase of transduction frequency was observed in FLYA13 and FLYRD18, suggesting that the receptors for these viruses on the surface membrane of human stem cells were saturated with the retroviruses with 3×10^5 CFU/ml. The data obtained from these transduction experiments indicated that while FLYRD18 showed the highest transduction efficiency in the T cell lineage, the packaging cell line best suited for stem cell transduction was PG13.

Table 3. Transduction frequency of $CD34^+$ cells

	Experiment 1[a]	Experiment 2[a]	Experiment 3[b]
PA317/G1NS	2.3	10.1	31.4
PG13/G1NS	38.0	27.3	26.4
FLYA13/G1NS	8.6	15.0	10.0
FLYRD18/G1NS	5.0	12.8	15.6

Transduction frequency (%) is determined by division of the colony number of the culture with G418 (1 mg/ml) by that of the culture without G418.

[a] All virus supernatants diluted to 3×10^5 CFU/ml prior to transduction.

[b] Undiluted supernatant used for transduction: PA317: 8×10^5, PG13: 3×10^5, FLYA13, FLYRD18: 7×10^6 CFU/ml.

Future Prospects

Although the present study indicates that PG13/ADA(MPSV) was more beneficial for clinical use, the final conclusion as to the effectiveness of the vector should be made only in clinical trials because in vitro results are often not predictive of success in clinical trials. We plan the next clinical gene therapy trial to determine which virus envelope is best suited for human hematopoietic stem cells. In this trial we will use three different packaging cell lines including ψCRIP with amphotropic env, PG13 with gibbon ape leukemia virus env and FLYRD18 with feline virus env. Furthermore, to minimize the effect of differences due to individual patient variation, we will divide the CD34+ cells into two groups, transducing each group with vectors from different packaging cell lines separately, mix them, and reinfuse this mixture into the patient (fig. 2c). Retroviruses from the three packaging cell lines can be distinguished by PCR since the vectors contain a 10-bp nucleotide difference downstream of the ADA gene. Results obtained from this clinical trial should demonstrate the best viral envelope for human hematopoietic stem cells.

It is very clear that current retroviral technology needs to be improved to make stem cell gene therapy applicable to the treatment of fatal disorders including congenital immunodeficiency and cancer. Nonetheless, the potential benefits of being able to perform specific genetic manipulations for the treatment of numerous diseases are great. With efforts to analyze human hematopoiesis and improve vector configurations, it is likely that gene transfer will have a significant clinical impact in the near future.

Acknowledgment

We thank Dr. W. Jay Ramsey, Dr. Linda M. Muul and Dr. Richard A. Morgan for helpful discussion.

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